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Degradation of phenol by a defined mixed culture immobilized by adsorption on activated carbon and sintered glass

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Summary. A defined mixed culture of the yeast *Cryptococcus elinovii* H1 and the bacterium *Pseudomonas putida* P8 was immobilized by adsorption on activated carbon and sintered glass, respectively. Depending on its adsorption capacity for phenol the activated carbon system could completely degrade 17 g/l in batch culture, whereas the sintered glass system was able to degrade phenol up to 4 g/l. During semicontinuous degradation of phenol (1 g/l) both systems reached constant degradation times with the fourth batch that lasted 8 h when using the activated carbon system and 10 h in the sintered glass system. In the course of continuous degradation of phenol the activated carbon system reached a maximum degradation rate of 9.2 $g\,l^{-1}\,day^{-1}$ compared to 6.4 $g\,l^{-1}\,day^{-1}$ degraded by the sintered glass system. 2-Hydroxymuconic acid semialdehyde could be identified and quantitatively determined as a metabolite of phenol degradation by *P. putida* P8. Increased membrane permeability under the influence of phenol was demonstrated by the examination of K^+ efflux from *P. putida* P8.

Introduction

In earlier investigations (Ehrhardt and Rehm 1985; Mörsen and Rehm 1987) *Cryptococcus elinovii* H1 and *Pseudomonas putida* P8 were adsorbed on activated carbon D 45/2 in pure and mixed cultures. These biocatalysts were used to degrade phenol in batch culture; 17 g/l could be completely degraded only by the mixed culture (Mörsen and Rehm 1987). *P. putida* P8 adsorbed on activated carbon D 45/2 reached a maximum degradation rate of 8.5 $g\,l^{-1}\,day^{-1}$ during continuous degradation of phenol (Ehrhardt and Rehm 1989).

Open-pored sintered glasses were specifically developed as a carrier for the immobilization of microorganisms and used with success in anaerobic waste-water treatment a few years ago (Aivasidis and Wandrey 1984). The open-pored structure secures particle transport without limitations and low fluid resistance.

The properties and limits of activated carbon and sintered glass as carriers for the immobilization of microorganisms have been investigated using as an example the microbial degradation of phenol. Phenol at low concentration acts as a membrane-active agent that increases permeability of the cytoplasmic membrane and so causes leakage of cytoplasmic material such as K^+, purines, pyrimidines, and amino acids (Hugo and Russell 1977). Leakage of K^+ as a first indication of membrane injury was shown to be proportional to the phenol concentration (Keweloh et al. 1990).

Materials and methods

Microorganisms. *C. elinovii* H1 and *P. putida* P8 were isolated from phenol containing municipal waste-water and used in earlier investigations (Ehrhardt and Rehm 1985, 1989; Mörsen and Rehm 1987; Mörsen et al. 1988). Phenol is degraded by *C. elinovii* H1 via the ortho-pathway, wheres *P. putida* P8 uses meta-cleavage (Mörsen 1990).

Mineral medium. The mineral salt medium was composed of the following components (g/l): K_2HPO_4, 1.0; NH_4NO_3, 1.0; KH_2PO_4, 0.5; $MgSO_4$, 0.5; NaCl, 0.5; $(NH_4)_2SO_4$, 0.5; $CaCl_2 \cdot 2\,H_2O$, 0.02; $FeSO_4 \cdot 7\,H_2O$, 0.02; 2 ml trace element solution (Pfennig and Lippert 1966). The final pH was 6.9. Phenol was added after sterilization.

Carrier materials. Activated carbon D 45/2 (Bergbau-Forschung, Essen, FRG) and sintered glass beads (Schott Glaswerke, Mainz, FRG) were used as carriers for immobilization of *C. elinovii* H1 and *P. putida* P8. Their characteristics are described in detail in Table 1.

Analytical methods. Phenol was determined quantitatively by the photometric method of Martin (1949) using 4-aminoantipyrene as the colour reagent. Process parameters such as pH and O_2 saturation were measured by means of standard laboratory equipment.

Optical density (OD) was measured at 560 nm to follow outgrowth of the microorganisms during fermentation. The ratio of yeasts to bacteria was determined by counting the cells in Thoma counting chambers.

Formation of 2-hydroxymuconic acid semialdehyde was fol-

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Table 1. Characteristics of the carrier activated carbon D 45/2 and sintered glass beads (Schott 1988)

Characteristics	Activated carbon	Sintered glass
Type/material	D 45/2	Siran glass
Particle size (cm)	0.2 × 0.4	Ø 0.3 to 0.5
Pore diameter:		
Macropores	> 50 nm: 60%	60–300 µm
Mesopores	2 –50 nm: 6%	
Micropores	0.3– 2 nm: 34%	1–10 µm
Porosity	No specification	55%
BET-surface (m^2/g)	1150	0.2
Bulk density (g/l)	450	490
Adsorption of phenol	Yes	No

lowed by measuring absorption at 375 nm. Samples were centrifuged to remove cells and made alkaline (pH > 8) by the addition of 2 *N* NaOH. The concentration of semialdehyde in the medium was calculated using the law of Lambert-Beer. The molar extinction coefficient of 2-hydroxymuconic acid semialdehyde is 33 000 $l\ mol^{-1}\ cm^{-1}$ at pH 8 (Nozaki et al. 1963).

In order to examine the K^+ efflux of *P. putida* P8 cells were grown in the mineral medium containing phenol or glucose (1 g/l). Cells were separated from the medium by centrifugation through silicone oil (Density = 1.05, Roth, Karlsruhe, FRG) as described by Bakker and Mangerich (1981). Intracellular K^+ was assayed after cell disruption in trichloroacetic acid (5%) by flame photometry.

Immobilization and experimental apparatus. C. elinovii H1 and *P. putida* P8 were obtained from separate precultures (23° C, 60 h) using the mineral medium which contained 1 g phenol/l. The number of adsorbed cells was estimated by determining the difference of the cell titres before and after immobilization.

For batch-cultures a fixed-bed loop reactor with a working volume of 200 ml was used for immobilization and fermentation. They were carried out with 10 g carrier at room temperature and an aeration rate of 5 vvm. Immobilization of *C. elinovii* H1 and *P. putida* P8 was performed simultaneously for about 12 h in the running loop reactor with 200 ml (each 100 ml, ratio 1:1) cell suspension.

For semicontinuous cultivation using activated carbon the adsorption capacity of the carbon for phenol had to be reduced in order to make the degradation capacities of both systems comparable. This was achieved by a biofilm developed during three fermentations on glucose (1 g/l mineral medium) as carbon source.

For continuous degradation of phenol immobilization of *C. elinovii* H1 and *P. putida* P8 was carried out separately in beakers using 300 ml cell suspension (ratio 1:1).

Table 2. Degradation of phenol by the activated carbon and sintered glass systems

	Degradation time (h)	
Phenol (g/l)	Activated carbon	Sintered glass
1	Not detectable	27
2	Not detectable	42
4	9	244
5	10	No degradation
10	80	No degradation
15	140	No degradation
17	218	No degradation
20	No degradation	No degradation

The carriers (six portions of 10 g) with adsorbed microorganisms were enveloped in plastic network (mesh size 1 mm) and put with space holders into the glass column reactors (working volume 600 ml). Aeration (2.5 vvm) was provided through a glass frit at the bottom, and the working temperature was 25° C. The phenol concentration in the feed was 1 or 2 g/l.

The tolerance against phenol shocks was investigated after finishing continuous cultivations on phenol, and degradation was performed in batch culture.

Scanning electron microscopy. The samples were fixed with glutaraldehyde and dehydrated in ethanol. After a critical point drying they were covered with a gold layer; for a detailed description see Mörsen and Rehm (1987).

Results

Adsorption of cells on activated carbon D 45/2 and sintered glass beads

Using identical starting cell titres ($1.5 \cdot 10^8$ cells/ml) *C. elinovii* H1 and *P. putida* P8 were adsorbed in different

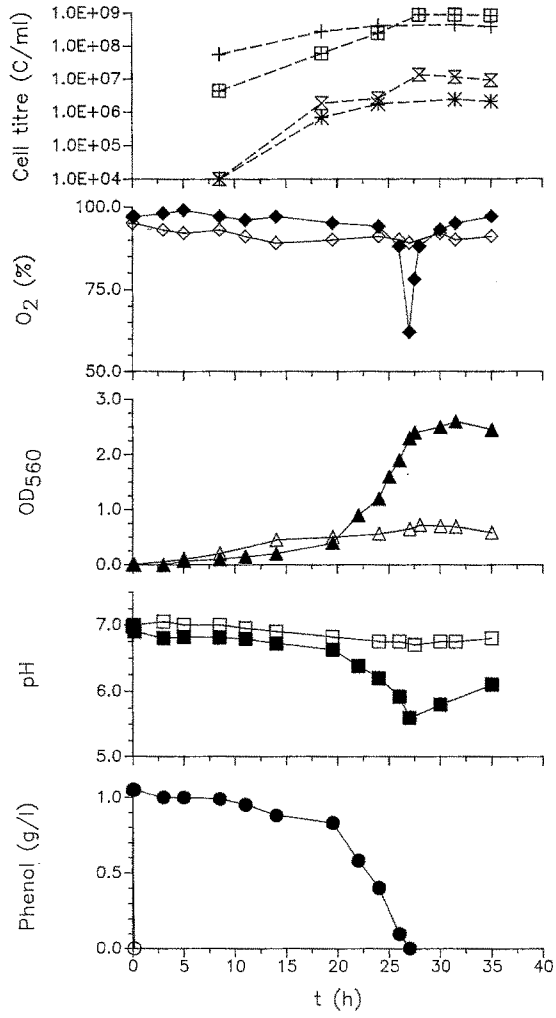

Fig. 1. Degradation of 1 g phenol/l by the activated carbon and sintered glass systems in batch culture. Activated carbon system: ○, □, △, ◇; *Cryptococcus elinovii* H1, *; *Pseudomonas putida* P8, +. Sintered glass system: ●, ■, ▲, ◆; *C. elinovii* H1, ×; *P. putida* P8, ⊞. OD_{560} = optical density at 560 nm

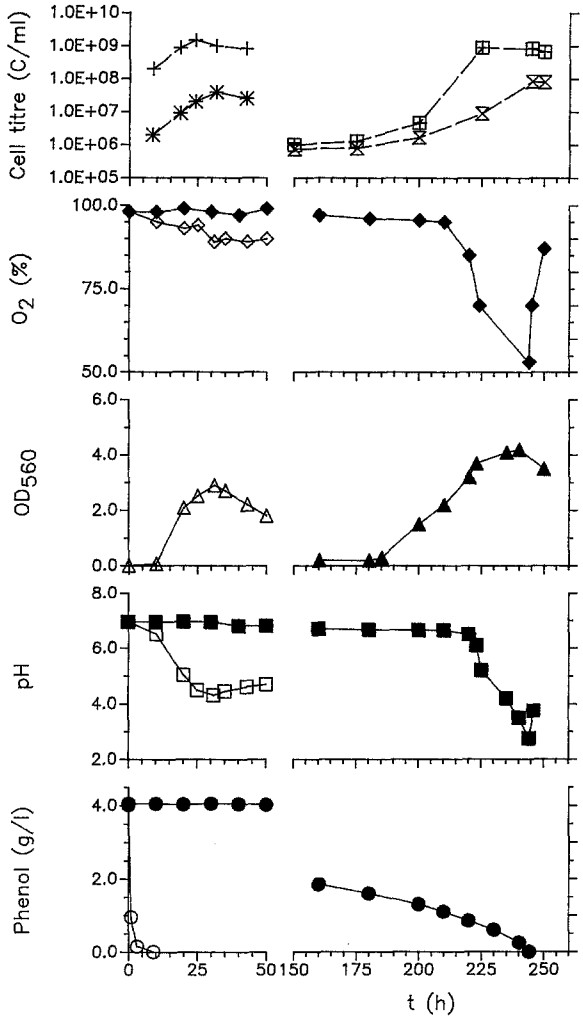

Fig. 2. Degradation of 4 g phenol/l by the activated carbon and sintered glass systems in batch culture. For symbols see Fig. 1 legend

numbers on the carriers: 79% yeasts ($1.2 \cdot 10^9$ cells/g) and 71% ($1.1 \cdot 10^9$ cells/g) bacteria were adsorbed on activated carbon, corresponding to a yeasts-to-bacteria ratio of 1.1:1. on the other hand 94% yeasts ($1.4 \cdot 10^9$ cells/g) but only 55% bacteria ($8.3 \cdot 10^9$ cells/g) were adsorbed on sintered glass beads. In this system the ratio was 1.7:1.

Degradation of phenol in batch culture

In order to study the properties of the activated carbon and sintered glass systems they were used to degrade different concentrated phenol solutions. Activated carbon D 45/2 has a high adsorption capacity for phenol (1 g adsorbs 0.7 g phenol) and for that reason phenol below 4 g/l is adsorbed within a short time (Ehrhardt and Rehm 1985; Mörsen and Rehm 1987). The activated carbon system was therefore able to degrade phenol much more quickly and in higher concentrations than the sintered glass system. The maximum phenol concentration that could be degraded by the activated carbon system was 17 g/l in contrast to 4 g/l tolerated by the sintered glass system (Table 2).

A comparison of degradation kinetics (1 and 4 g phenol/l, batch cultures) of both systems is shown in Figs. 1 and 2. In case of the activated carbon system the degradation kinetics of 1 g phenol/l could only be determined by following the pH and OD at 560 nm (OD_{560}). Phenol should be completely degraded by the adsorbed and grown-out microorganisms when the OD stopped increasing. The sintered glass system showed a more drastic decrease in pH and oxygen saturation just at the moment when phenol could not longer be detected in the medium. While degrading 4 g phenol/l the pH of the sintered glass system reached 2.75 compared to 4.3 using the activated carbon system.

Semicontinuous cultivation

Both systems could be used very well for semicontinuous degradation of phenol (1 g/l). Constant degradation times of 8 h using the activated carbon system and 10 h using the sintered glass system were reached with the fourth batch. The adsorption of phenol to the activated carbon was rapidly reduced by a biofilm (Fig. 3).

Continuous degradation of phenol

The maximum degradation rate of the activated carbon system was 9.2 $g\,l^{-1}\,day^{-1}$ at a dilution rate (D) of 0.2 h^{-1} reached after 393 h. The sintered glass system only reached 6.4 $g\,l^{-1}\,day^{-1}$ after 360 h at $D = 0.14\ h^{-1}$ (Fig. 4). During continuous degradation of phenol the same effects concerning pH and OD as in batch cultures could be observed. The pH of the activated carbon system only decreased to 5.1 in contrast to 4.3 reached by the sintered glass system. In this system the OD was always two to three times higher than using the sintered glass system. On the other hand biomass build-up on the carriers determined by lyophilization after

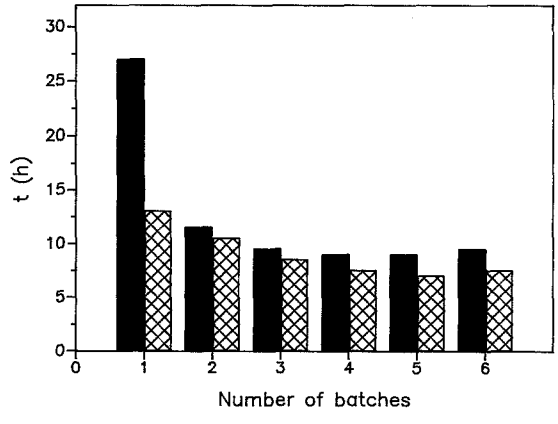

Fig. 3. Semicontinuous degradation of phenol (1 g/l) by the activated carbon and sintered glass systems. Activated carbon system, ▨; sintered glass system, ■

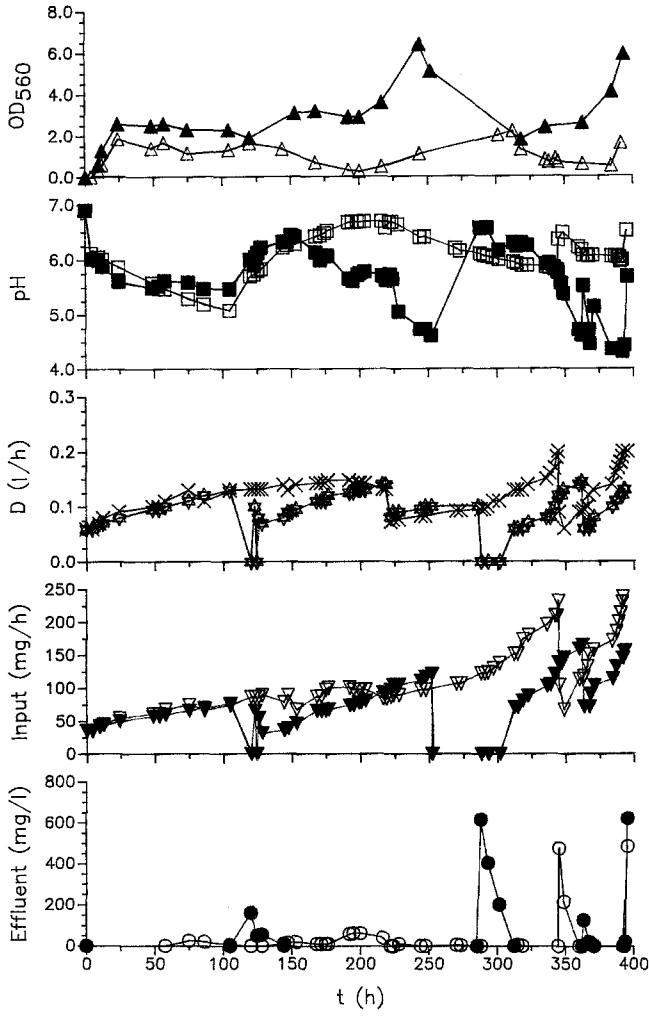

Fig. 4. Continuous degradation of phenol by the activated carbon and sintered glass systems. Activated carbon system: ○, □, △, ▽, ×; sintered glass system: ●, ■, ▲, ▼, ; →, the phenol concentration in the feed was raised to 2 g/l; D = dilution rate

fermentations finished was 6 g for the activated carbon system but only 1 g on the sintered glass beads.

Using the activated carbon system *C. elinovii* H1 only grew out during the first 10 days of fermentation while with the sintered glass system yeasts could be observed in the medium all the time. At the end of the experiments the following cell titres of grown-out microorganisms were achieved: activated carbon system, $2.5 \cdot 10^8$ *C. elinovii* cells/ml; sintered glass system, $7.2 \cdot 10^5$ cells *C. elinovii* H1 and $1.8 \cdot 10^9$ cells of *P. putida* P8 per ml.

Table 3. Degradation of phenol shocks by the activated carbon and sintered glass systems

Phenol (g/l)	Degradation time (h)	
	Activated carbon	Sintered glass
1	2	10
2	7	44
5	28	No degradation
8	50	No degradation
10	146	No degradation
12	No degradation	No degradation

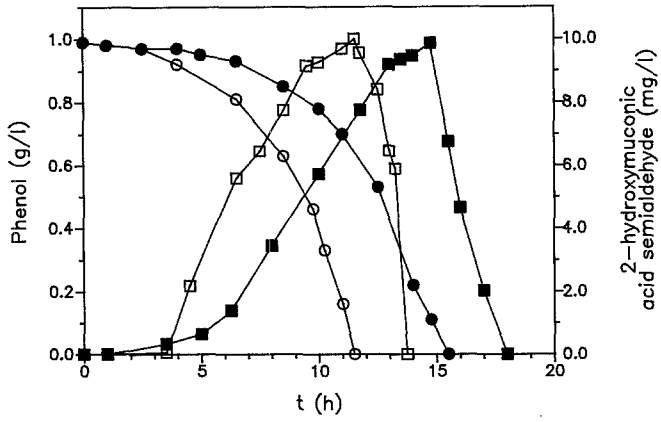

Fig. 5. Formation of 2-hydroxymuconic acid semialdehyde by *P. putida* P8 and in mixed culture with *C. elinovii* H1 adsorbed on sintered glass beads during degradation of phenol (1 g/l): phenol levels with *P. putida* P8, ● — ●; phenol levels with the mixed culture, ○ — ○; 2-hydroxymuconic acid semialdehyde levels with *P. putida* P8, ■ — ■; 2-hydroxymuconic acid semialdehyde levels with the mixed culture, □ — □

Tolerance against phenol shocks

Phenol shocks up to 10 g/l could be tolerated and the phenol was completely degraded by the activated carbon system. In contrast to this only 2 g phenol were degraded by the sintered glass system (Table 3).

Identification and formation of 2-hydroxymuconic acid semialdehyde

During growth of free cells of *P. putida* P8 on phenol the colour of the medium changes to an intensive not fluorescing green-yellow. The same effect was also observed when *P. putida* P8 in a pure culture or together with *C. elinovii* H1 was adsorbed on sintered glass

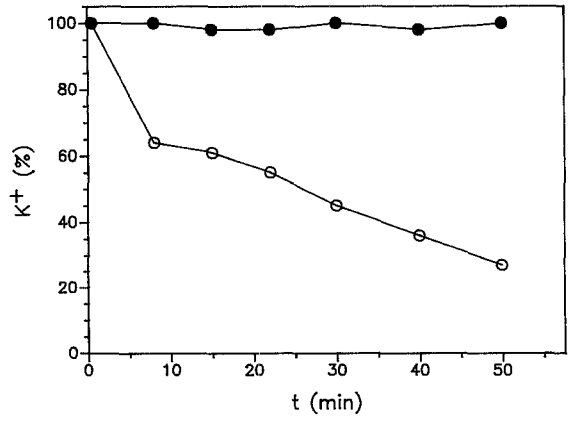

Fig. 6. Potassium (K^+) efflux from *P. putida* P8 grown on phenol (○ — ○) or grown on glucose (● — ●)

beads. Always the colour reached its maximum at the end of phenol degradation. Absorption spectra with cell-free solutions were taken at different pH values (for details see Mörsen 1990). At alkaline pH the absorption maxima lay at 375 nm. These results are in a good agreement with data reported by Dagley et al. (1960) and Sala-Trepat and Evans (1971) for 2-hydroxymuconic acid semialdehyde, a metabolite of the meta-pathway degradation of phenol.

Figure 5 shows the formation of 2-hydroxymuconic acid semialdehyde during phenol degradation (1 g/l) by the sintered glass system and *P. putida* P8 in a pure culture adsorbed on sintered glass beads, respectively. In both experiments the concentration of the semialdehyde reached its maximum at about 10 mg/l just at the moment when phenol could not be detected in the medium any longer. After 3 h 2-hydroxymuconic acid semialdehyde was also no longer detectable. The nearly

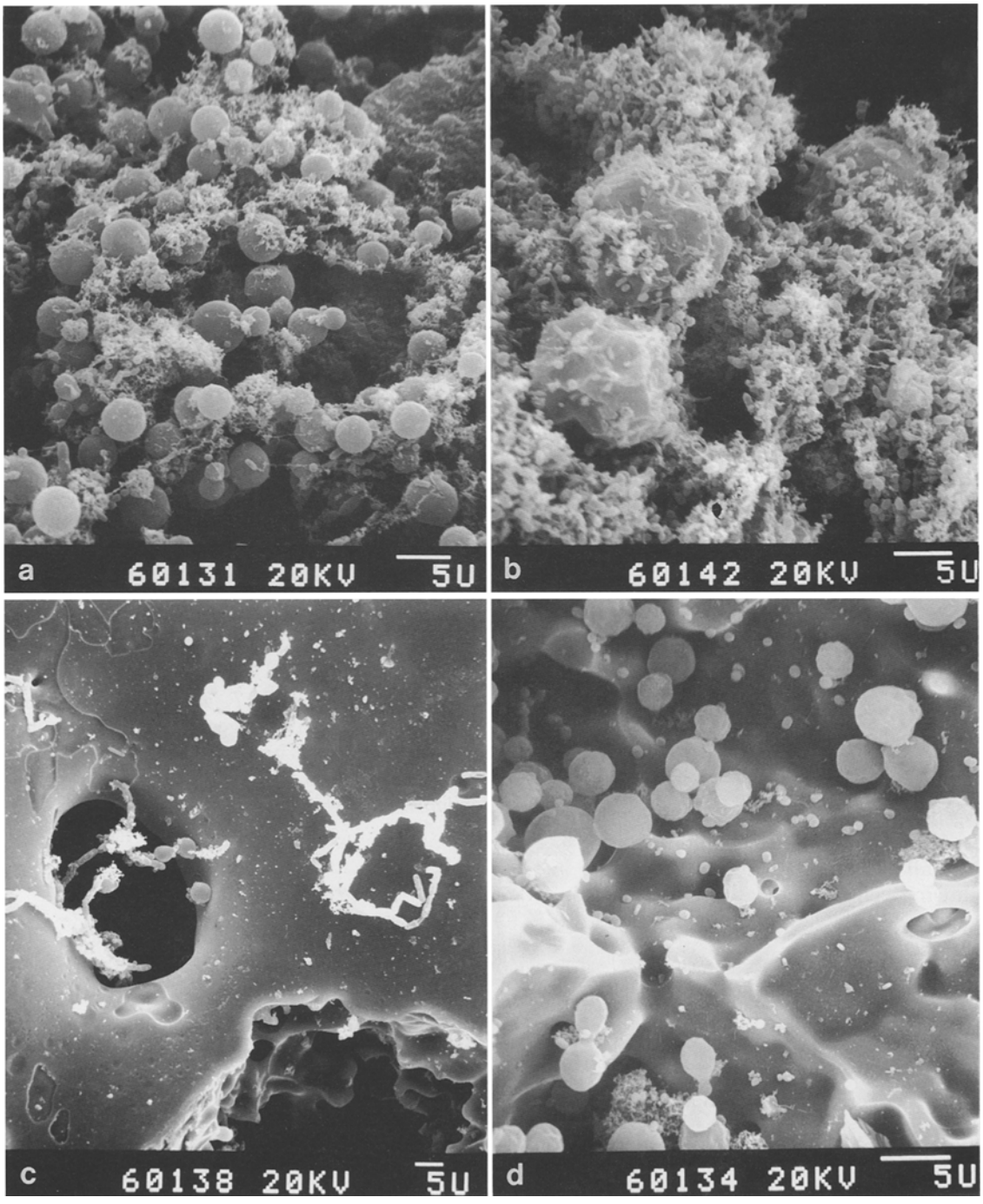

Fig. 7. *C. elinovii* H1 and *P. putida* P8 adsorbed on activated carbon after degradation of 4 g phenol/l (**a**); activated carbon after continuous degradation of phenol (**b**); sintered glass beads after degradation of 4 g phenol/l (**c**); sintered glass beads after continuous degradation of phenol (**d**)

identical development of the kinetics shown in Fig. 5 proves that *P. putida* P8 alone was responsible for the formation of 2-hydroxymuconic acid semialdehyde.

K^+ efflux of P. putida

In order to investigate the increased membrane permeability caused by phenol, the K^+ efflux of *P. putida* P8 cells was measured. Only cells grown on phenol showed a drastic loss of K^+: 75% of the total K^+ content was lost during the 50-min experiment. On the other hand cells grown on glucose did not lose K^+ (Fig. 6).

Morphological observations

Scanning electron micrographs were taken from samples prepared after degradation of 4 g phenol/l and continuous cultivation on phenol, respectively. In both cases activated carbon particles showed a thick biofilm mainly built up by *P. putida* P8 (Fig. 7a and b). Cells of *C. elinovii* H1 were grown over by the bacteria during continuous cultivation on phenol (Fig. 7b). Sintered glass beads only showed isolated accumulations of *C. elinovii* H1 and *P. putida* P8 even after continuous degradation of phenol (Fig. 7c and d).

Discussion

A great advantage of activated carbon compared to sintered glass is the ability to work as a buffer by adsorbing high phenol concentrations immediately and so protect adsorbed microorganisms against damage by phenol (Ehrhardt and Rehm 1985, 1989; Mörsen and Rehm 1987). This effect became especially clear during degradation of phenol in batch culture and in the case of phenol shocks, where adsorption of high phenol concentrations on activated carbon was not influenced even if a biofilm was built up around the carbon particles. On the other hand nearly comparable degradation capacities of the activated carbon and sintered glass systems were achieved during semicontinuous and continuous cultivation when low phenol concentrations were used. In these experiments much active biomass that quickly degraded phenol was present in the reactor, so adsorption of phenol by the activated carbon could not occur. Comparable results were reported by Ehrhardt and Rehm (1989).

Using the sintered glass system in batch and continuous cultures a much more drastic decrease in pH was observed compared to the activated carbon system. This pH equalizing effect of activated carbon is caused by acidic and basic surface oxides that influence the pH of a solution (Mattson and Mark 1971).

Prober et al. (1975) showed that activated carbon is able to enrich dissolved oxygen. Probably this oxygen can also be utilized by microorganisms adsorbed on activated carbon. This would explain the observation that O_2 saturation during degradation of 4 g phenol/l only decreased to 90% when using the activated carbon system in contrast to the sintered glass system where it decreased to 52%.

Examinations on the K^+ efflux of *P. putida* P8 cells grown on phenol showed a loss of 75% intracellular K^+ after 50 min. The permeability of the cytoplasmic membrane was increased by phenol. Probably 2-hydroxymuconic acid semialdehyde as a low molecular substance can pass through the damaged membrane.

Biomass built up on activated carbon during continuous degradation of phenol was six times higher than on sintered glass beads. Also scanning electron micrographs showed activated carbon particles covered by a biofilm in contrast to sintered glass beads where only isolated accumulations of microorganisms were observed. One reason might be the different surface structures of the carriers: activated carbon shows a rough and fissured surface where microorganisms can settle and colonize easily. The surface of the sintered glass beads appears smoother, and the diameters of the pores are mostly greater. The relationship between pore diameter and maximum accumulation of stable biomass has been described by Messing et al. (1979): the optimal pore diameter of a carrier should be up to four times (yeasts) and five times (bacteria) larger than the cells themselves.

Because of the results described above, activated carbon seems to be better suited to serve as a carrier for the immobilization of microorganisms in the treatment of waste-waters where high concentrations of pollutants are expected. On the other hand inert and non-adsorbing carriers such as glass may be preferred in anaerobic processes or biotechnological production. For example, good results were achieved in the semicontinuous and continuous production of glycerol by *Saccharomyces cerevisiae* cells adsorbed on sintered glass Raschig rings (Hecker et al. 1989).

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